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c-Src Is Required for Tropomyosin Receptor Kinase C (TrkC)-induced Activation of the Phosphatidylinositol 3-Kinase (PI3K)-AKT Pathway*□S

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TrkC mediates many aspects of growth and development in the central nervous system. TrkC is expressed in a variety of non-neuronal tissues as well as human cancers. TrkC overexpression may drive tumorigenesis, invasion, and metastatic capability in cancer cells. However, relatively little is known about whether TrkC activity is also essential to maintain the malignant properties in human tumors. TrkC expression leads to the constitutive activation of two major effector pathways, namely the Ras-MAP kinase (MAPK) mitogenic pathway and the phosphatidylinositol 3-kinase (PI3K)-AKT pathway mediating cell survival. However, it remains unclear how TrkC activates Ras-Erk1/2 and/or PI3K-Akt cascades. Here we define some aspects of the molecular mechanisms regulating TrkC-dependent Ras-Erk1/2 and PI3K/Akt activation. We show that endogenous TrkC associated with c-Src in human and mouse cancer cells which express TrkC. TrkC-c-Src complexes were also detected in primary human breast cancer tissues. Suppression of c-Src by RNA interference in highly metastatic 4T1 mammary cancer cells, which express endogenous TrkC, resulted in markedly decreased expression of cyclin D1 and suppression of activation of Ras-Erk1/2 and PI3K-Akt. Moreover, inhibition of c-Src expression almost completely blocks colony formation of 4T1 cells in soft agar. Furthermore, in c-Src-deficient SYF cells, TrkC failed to activate the PI3K-Atk pathway, but not the Ras-Erk1/2 pathway. Therefore these data indicate that TrkC induces the PI3K-Akt cascade through the activation of c-Src.

Neurotrophins constitute a family of structurally related growth factors that regulate growth, differentiation, and survival of neurons (1–4), as well as in some non-neuronal cells (5–7). This family includes nerve growth factor (NGF),[2] brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4 (1, 2). Receptors for neurotrophins include the Trk family of receptor-tyrosine kinases (RTKs). TrkA preferentially binds NGF, TrkB binds BDNF, and neurotrophin-4/5, and TrkC binds neurotrophin-3 (8). Neurotrophins and their corresponding receptors have been shown to induce a variety of pleiotropic responses in malignant cells, including enhanced tumor invasiveness and chemotaxis (9–13). Also, neurotrophins and their receptors are important in the regulation of angiogenesis (14), mitogenic signals, facilitating tumor growth (15), prevention of apoptosis (16), and cell spreading and metastasis (17). Trk receptors are frequently overexpressed in pancreatic tumors, thyroid tumors, breast tumors, lung tumors, melanomas, and prostatic tumors (11, 13, 18–27).

TrkC mediates many aspects of growth and development in the central nervous system (13). A variety of non-neuronal tissues have been shown to express TrkC (13, 27–30); however, relatively little is known about whether TrkC activity is also essential to maintain the malignant properties in human tumors. Interestingly, recent sequence analysis of the tyrosine kinome in colorectal cancers has revealed five point mutations in the *trkC* gene, both within its kinase-encoding domain (9). The effect of the *trkC* mutations on kinase function remains to be determined, but their positions within each protein suggest that many of them may be activating in nature.

TrkC is activated by cell surface ligand-mediated oligomerization, which facilitates autophosphorylation of TrkC cytoplasmic tyrosine residues 516, 705, 709, 710, and 820 and subsequent kinase activation (31–33). Several adapter molecules and protein-tyrosine kinase (PTK) substrates have been linked to TrkC signal transduction. Phosphorylated TrkC tyrosine 516 binds the adaptor protein Src-homology/collagen (SHC) via the SH2 domain of the latter (34), and is also the site of association with the p85 subunit of phosphoinositol 3-kinase (PI3K) (31, 35). Activated SHC recruits growth factor receptor-bound protein 2 (GRB2), an adaptor protein, as well as the RAS guanine nucleotide exchange factor Son-of-sevenless (Sos) to activate the RAS-RAF1 pathway (36, 37). Phosphorylated PI3K mediates activation of the protein kinase B (Akt/PKB) pathway (38).

[2] The abbreviations used are: NGF, nerve growth factor; TrkC, tropomyosin receptor kinase C; EN, ETV6-NTRK3; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; IB, immunoblot; IP, immunoprecipitation; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MSCV, murine stem cell virus; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PTB, phosphotyrosine binding domain.

TABLE 1

siRNAs sequences for the mouse *c-src* gene and the mouse *IRS-1* gene

Mouse c-Src-1 (pFG12 vector)
5′-CTAGACC<u>AGCCGCCAATATCCTAGT</u>ATTCAAGAGA<u>TACTAGGATATTGGCGGC</u>TTTTTGGAAAC-3′
Mouse c-Src-2 (pFG12 vector)
5′-CTAGACC<u>AAGATCACTAGACGGGAATCA</u>TTCAAGAGA<u>TGATTCCCGTCTAGTGATC</u>TTTTTTTGGAAAC-3′
Mouse IRS-1 515 (pFG12 vector)
5′-CTAGACC<u>AGAACCTGATTGGCATCT</u>ATTCAAGAGA<u>TAGATGCCAATCAGGTTC</u>TTTTTTGGAAAC-3′
Mouse IRS-1 1539 (pFG12 vector)
5′-CTAGACC<u>TAACCGGTTTCGAAAGAGA</u>TTCAAGAGA<u>TCTCTTTCGAAACCGGTTA</u>TTTTTGGAAAC-3′

Phosphorylated NTRK3 tyrosine 820 binds phospholipase-Cγ (PLCγ) (39), resulting in the activation of protein kinase C (PKC) (39–41).

The congenital fibrosarcoma t(12, 15)(p13;q25) rearrangement splices the *ETV6* (*TEL*) gene on chromosome 12p13 in-frame with the *NTRK3* (*TrkC*) neurotrophin-3 receptor gene on chromosome 15q25. Resultant *ETV6-NTRK3* fusion transcripts encode the helix-loop-helix (HLH) dimerization domain of ETV6 fused to the protein-tyrosine kinase (PTK) domain of NTRK3. Expression of the ETV6-NTRK3 fusion protein is associated with constitutively high levels of phosphorylated Mek1 and Akt, even in the absence of serum. Moreover, ETV6-NTRK3-expressing cells show serum-independent elevation of cyclin D1 protein. Inhibition of either the Ras-Erk1/2 MAP kinase or the PI3K-Akt pathway alone completely blocks colony formation of ETV6-NTRK3-expressing cells in soft agar assays. Furthermore, the constitutive expression of cyclin D1 protein in ETV6-NTRK3-expressing cells can be down-regulated by MEK1 or PI3K inhibition. However, only inhibition of the Ras-Erk1/2 pathway led to persistent down-regulation of cyclin D1 levels in cells expressing ETV6-NTRK3 (42).

c-Src is known to activate the PI3K/Akt and the Ras-MAPK cascades through both FAK-dependent and FAK-independent pathways (43, 44). c-Src has also been implicated, in PC12 cells, in NGF-induced down-regulation of EGF receptors. Expression of dominant-negative c-Src blocked the tyrosine phosphorylation of mitogen-activated protein kinases (45). These results prompt us to examine whether TrkC activates Ras-MAPK and PI3K/Akt through the activation of c-Src. Here we show that TrkC binds to and activates c-Src, and that inhibition of c-Src activation blocks TrkC transforming activity and activation of the PI3K/Akt and the Ras-MAPK cascades.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies and Reagent—Mouse embryonic fibroblasts (MEF) deficient in c-Src, Yes, and Fyn (SYF) as well as c-Src (SYF cells deficient for Yes and Fyn but overexpressing c-Src) cells were obtained from the ATCC. 67NR, 4T1, 293T, and SYF cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Expression of Src was frequently assessed by Western blot analysis. Antibodies were obtained from the following companies: Anti-c-Src, anti-TrkC, anti-HA(Y-11), anti-cyclin D1, and anti-Myc(9E10) from Santa Cruz Biotechnology; anti-V5 from Invitrogen; anti-phospho-Src, anti-phospho-MEK1/2, and anti-phospho-AKT from Cell Signaling Technology; anti-β-actin from Sigma-Aldrich. Pharmacological inhibition was performed with the PI 3-kinase inhibitor LY294002 (20 μM) and c-Src family tyrosine kinase inhibitor SU6656, both from Calbiochem.

Plasmids—Three siRNA-coding oligos against mouse c-Src and *IRS-1* were designed and verified to be specific to c-*src* and *IRS-1* by Blast search against the mouse genome. To construct hairpin-type single RNAi vectors, 5 μl (100 mM) of the synthesized sense and antisense oligonucleotides (Table 1) were combined with 1 μl of 1 M NaCl and annealed by incubation at 95 °C for 2 min, followed by rapid cooling to 72 °C, and ramp cooling to 4 °C over a period of 2 h. The c-*src*-siRNA-1 and c-*src*-siRNA-2 insert were subcloned into the XbaI/XhoI sites of the pFG12 lentivirus vector. A control siRNA, which does not match any known mouse coding cDNA, was used as a control.

Immunoblotting and Immunoprecipitation—293T cells were used for the detection of protein-protein interaction *in vivo*. 293T cells were transiently transfected with the indicated plasmids. After a 24-h transfection, cells were switched to 0.2% serum overnight. Cells were lysed in a buffer containing 25 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and protease inhibitor mixture (Complete, Roche Applied Science, Gipf-Oberfrick, Switzerland). Extracts were separated by SDS/PAGE followed by electrotransfer to polyvinylidene difluoride membranes and probed with polyclonal or monoclonal antisera, followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgG, respectively, and visualized by chemiluminescence according to the manufacturer's instructions (Pierce). For immunoprecipitation, the cell lysates were incubated with the appropriate antibody (Ab) for 1 h, followed by incubation with Gamma-bind beads (Amersham Biosciences) for 1 h at 4 °C. Beads were washed four times with the buffer used for cell solubilization. Immune complexes then were eluted by boiling for 3 min in 2× Laemmli buffer (pH 6.8), and then extracts were analyzed by immunoblotting as described above.

Soft Agar Assays—Soft agar assays were performed according to established protocols (29). 67NR and 4T1 and 67NR expressing activated c-Src or 4T1 cells carrying siRNA-coding oligos against mouse c-Src and IRS-1 were seeded in triplicate at a concentration of 8×10^3 cells/well in a 6-well plate. Bottom layers were made up of 0.4% agar in 9% fetal bovine serum and DMEM. Cells were resuspended in a top layer of 0.2% agar in 9% fetal bovine serum and DMEM. Cells were fed every other day by placing 2 drops of medium on the top layer. After 3 weeks at 37 °C, colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/ml stock diluted at 1:500) for 16 h, and macroscopic colonies were counted (in quadruplicate). Each determination was done in triplicate.

Anchorage-independent Cell Growth— 67NR and 4T1 were seeded into an Ultra Low Cluster plate (ULC plate) (Corning
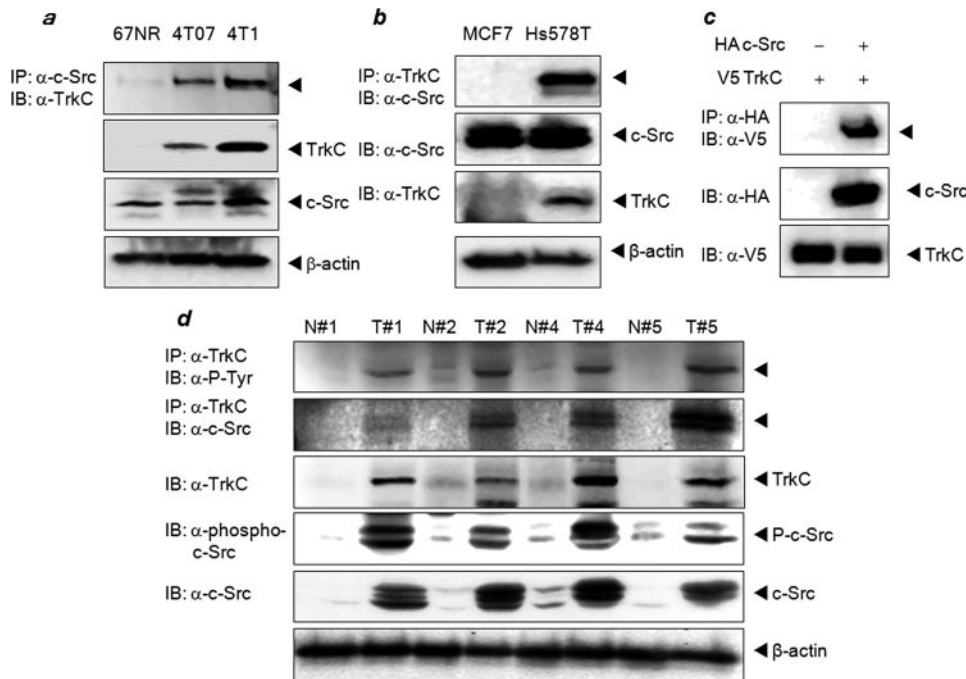
FIGURE 1. **TrkC interacts with c-Src.** The expression of c-Src or TrkC was monitored as indicated. Identification of endogenous TrkC-c-Src complexes in 4T07 and 4T1 cells (*a*) as well as in Hs578T cells (*b*) is shown. The cell lysates were subjected to immunoprecipitation using anti-TrkC Ab, followed by immunoblotting with anti-Src antibody. Whole cell lysates also were probed for TrkC, c-Src, and β-actin as a loading control. *c*, HA-tagged c-Src and V5-tagged TrkC were transfected into 293T cells. Cell extracts were subjected to immunoprecipitation using an anti-HA antibody and Gamma-bind beads, followed by immunoblotting with anti-V5 antibody. *d*, identification of TrkC-c-Src complexes in human primary tumors. The protein of normal and primary breast tumor tissue extracts from breast cancer patients were subjected to immunoblotting with anti-TrkC antibody and Gamma-bind beads, followed by immunoblotting with anti-c-Src, anti-phospho-c-Src, or anti-phosphotyrosine antibody. The endogenous β-actin level was measured as an internal control.

extracted using a Quizol reagent (Qiagen, Inc., Valencia, CA). Reverse transcription was done using a one step RT-PCR kit at the suggested conditions (Qiagen, Inc.). PCR products were separated on 1% agarose gels and visualized by ethidium bromide techniques.

RESULTS

TrkC Directly Binds to c-Src—We examined the possibility that TrkC may interact directly with c-Src *in vivo* in a mouse mammary model that consists of three distinct tumor cell lines, 67NR, 4TO7, and 4T1. These cell lines are derived from a single mammary tumor that arose spontaneously in a wild-type BALB/c mouse. 67NR cells form primary tumors, but no tumor cells are detectable in any distant tissue. 4TO7 cells are able to spread to the lung, but cannot establish visible metastatic nodules, whereas 4T1 cells are able to complete all steps of metastasis and form visible metastatic nodules in the lung efficiently (46). The 67NR cell line had negligible TrkC expression, but TrkC is expressed in the 4TO7 and

Life Sciences, Acton, MA) at 1×10^6 cells and photographed at 96 h.

Viral Production and Infection of Target Cells—293T cells were transfected with the transfer vector plasmid pCAG (empty) or pCAG-TrkC plasmid and pCAG-v-Src with the envelope-encoding plasmid pHCMVG, the packaging plasmid pMDLg/pRRE, and the Rev-expression plasmid pRSV-Rev using the calcium phosphate method. The supernatants were harvested 48 and 72 h after transfection, pooled, passed through a 0.45-μm filter, ultracentrifuged for 2 h at 100,000 $\times$ *g* in a SW28 rotor, resuspended in 100 μl of 0.1% bovine serum albumin in phosphate-buffered saline, and the lentiviral stocks were stored in small aliquots at -80 °C for titration and cell infection. SYF and SYF-Src cells were plated in 6-well plates (1×10^5 cells/well) and were cultured overnight. Lentiviruses were diluted in 2 ml of DMEM containing polybrene (8 μg/ml) and then centrifuged for 30 min at 1,500 rpm. After 24 h of infection, polybrene-DMEM was replaced with fresh DMEM medium, and the cells were cultured for other assays.

Reverse Transcription-PCRs—Mouse *c-src* RT-PCR forward primer is GCATCATCCCAGCCAACTAT, and its RT-PCR reverse primer is CAGCTGCATGAGGTTCTCAA. Mouse *IRS-1* RT-PCR forward primer is GGTGCTGCAGCT-GATGAATA, and its RT-PCR reverse primer is CGAGATCT-CCGAGTCAGTCC. Mouse *GAPDH* RT-PCR forward primer is AAGGTCATCCCAGAGCTGAA, and its RT-PCR reverse primer is CCCTGTTGCTGTAGC CGTAT. Total RNA was

4T1 cell lines (Fig. 1*a*). We have recently demonstrated that TrkC was expressed 5–10-fold higher in 4T1 tumor cells compared with 4TO7 cells and that TrkC proteins in 4TO7 and 4T1 cells are tyrosine-phosphorylated, indicating an activated state.[3] We determined the endogenous interaction of TrkC and c-Src in 4T07 and 4T1 cells, which express endogenous TrkC. To this end, we performed immunoprecipitation experiments in 4T07 and 4T1 cells. Extracts prepared from 67NR cells were used as negative controls. Endogenous TrkC was able to interact with c-Src (Fig. 1*a*). We also examined the endogenous interaction between TrkC and c-Src in Hs578T human breast cancer cells which express TrkC. MCF7 human breast cancer cells, which do not express TrkC, were used as a control. Endogenous c-Src was able to interact with endogenous TrkC (Fig. 1*b*). To confirm this finding in a transient transfection system, V5-tagged TrkC and HA-tagged c-Src were transfected into 293T cells. As shown in Fig. 1*c*, TrkC interacted strongly with c-Src. We then determined whether expression of TrkC correlates with certain pathological phenotypes in clinical breast tumor samples, and wished to confirm that the above findings are relevant to human breast tumors expressing TrkC. To do so, we examined TrkC expression in invasive human breast tumors by immunoblotting. Elevated expression of both TrkC and c-Src was observed in infiltrating ductal carcinomas compared with normal breast tissue samples (Fig. 1*d*), suggesting that

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[3] S.-J. Kim, submitted for publication.

expression of both TrkC and c-Src may contribute to the pathogenesis of human invasive breast carcinomas. We therefore screened for the presence of TrkC-c-Src complexes in human breast tumors positive for the *TrkC* gene. Total tissue extracts were isolated from primary tumors and subjected to immunoprecipitation with the anti-c-Src antibody followed by immunoblotting with the anti-TrkC antibody. As shown in Fig. 1*d*,
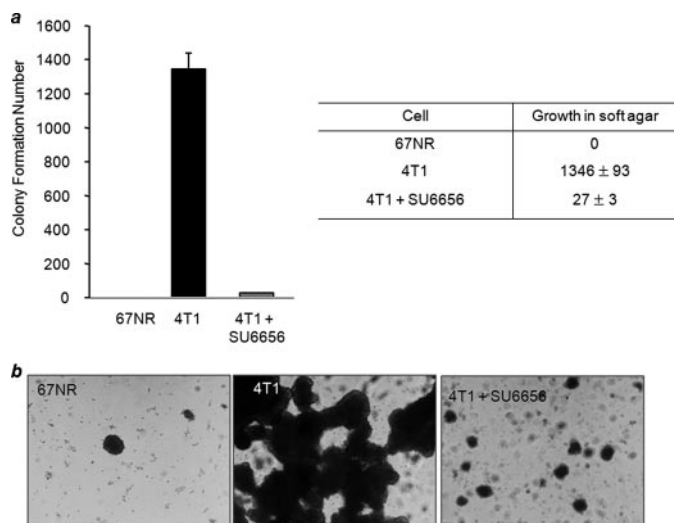

FIGURE 2. **Inhibition of c-Src activation blocks soft agar colony formation of 4T1 cells.** *a*, soft agar colony-forming assay of mouse breast cancer cell lines. Monolayer cells were trypsinized, washed, and plated in medium containing 0.2% agar to assess anchorage-independent growth in the presence or absence of SU6656 (1 μM). Results are presented as the number of macroscopic colonies formed at 3 weeks after plating. *b*, 4T1 mouse breast cell line as well as the control 67NR cell line were grown in medium containing 9% serum and were treated with vehicle control (dimethyl sulfoxide) and SU6656 (1 μM) for 3 days. Anchorage-independent spheroid growth of 67NR and 4T1 cells was then photographed in time at ×200 magnifications.
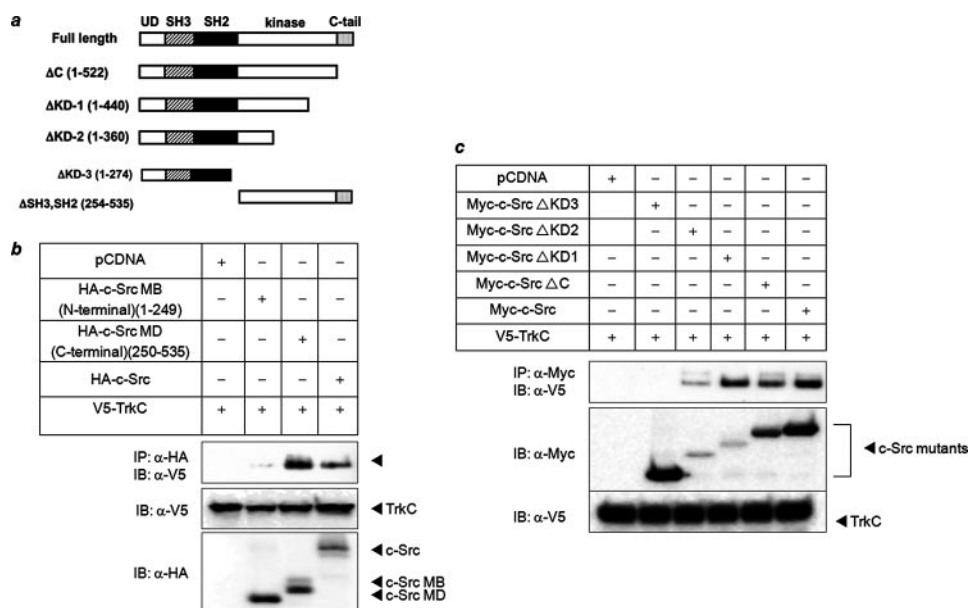

FIGURE 3. **Identification of c-Src region responsible for the interaction with TrkC.** *a*, schematic representation of full-length and truncated c-Src proteins. *b*, V5-tagged TrkC was cotransfected into 293T cells with the HA-tagged c-Src constructs. Cell extracts were subjected to immunoprecipitation using anti-HA antibody, followed by immunoblotting with anti-V5 antibody. The expression of c-Src or TrkC proteins was monitored as indicated. *c*, immunoprecipitation analyses of overexpressed V5-TrkC and Myc-tagged c-Src proteins in 293T cells.

TrkC-c-Src complexes were found in the human breast tumor samples, but not in control breast tissue samples. TrkC expression was associated with a strong increase in phosphorylation of c-Src. These results clearly demonstrate that the interaction between TrkC and c-Src occurs *in vivo* in human breast cancer tissues expressing the *TrkC* gene.

SU6656 Treatment Suppresses TrkC Transformation Activity—We have recently demonstrated that inhibition of TrkC expression completely blocks colony formation of 4T1 cells in soft agar.[3] This finding suggests that TrkC may induce the colony formation of 4T1 cells through the activation of c-Src. Therefore, we investigated whether the inhibition of c-Src kinase activity blocks the colony formation of 4T1 cells using SU6656 (47), an inhibitor of c-Src kinase. Upon the addition of 2 μM SU6656, the colony formation of 4T1 cells was almost completely suppressed indicated by the failure to form microscopic or macroscopic colonies in soft agar when scored after 16 days (Fig. 2*a*). Most tumor cell lines form multicellular spheroids through cell-cell adhesion on non-adhesive agar-coated dishes. Under these conditions, non-malignant cells generally fail to do so and undergo massive cell death in culture (48). 4T1 cells readily form multicellular spheroids (49); however, SU6656 treatment suppressed formation of multicellular spheroids (Fig. 2*b*). This result suggested that TrkC and c-Src affect the survival of 4T1 cells.

To see whether activation of c-Src can induce the colony forming ability, we generated the stable 67NR cells expressing v-Src, which is the constitutively active form of Src. Expression of v-Src in 67NR cells was verified by immunoblotting using the anti-V5 antibody (Supplemental Fig. S1*a*). And then we investigated whether v-Src increased the colony forming ability in these cells. Expression of v-Src markedly increased formation of microscopic or macroscopic colonies in soft agar when scored after 20 days (Supplemental Fig. S1*b*).

Characterization of the c-Src/TrkC Interaction—c-Src is a multidomain protein containing an unique N-terminal domain: UD (residues 1–80), SH3 (residues 83–144), SH2 protein interaction domains (150–247), a catalytic region (269–522), and a negative-regulatory tyrosine located near the C terminus (residue 523) (50–52) (Fig. 3*a*). To identify the functional domain of c-Src responsible for the interaction with TrkC, we used a series of c-Src deletion constructs. The c-Src mutant lacking the N-terminal domain (1–249) still interacted with TrkC (Fig. 3*b*). This indicates that the c-Src C-terminal region (250–535) is directly involved in this interaction. Next, we tested the interaction of TrkC with a series of C-terminal deletion mutants. The c-Src ΔC, which does
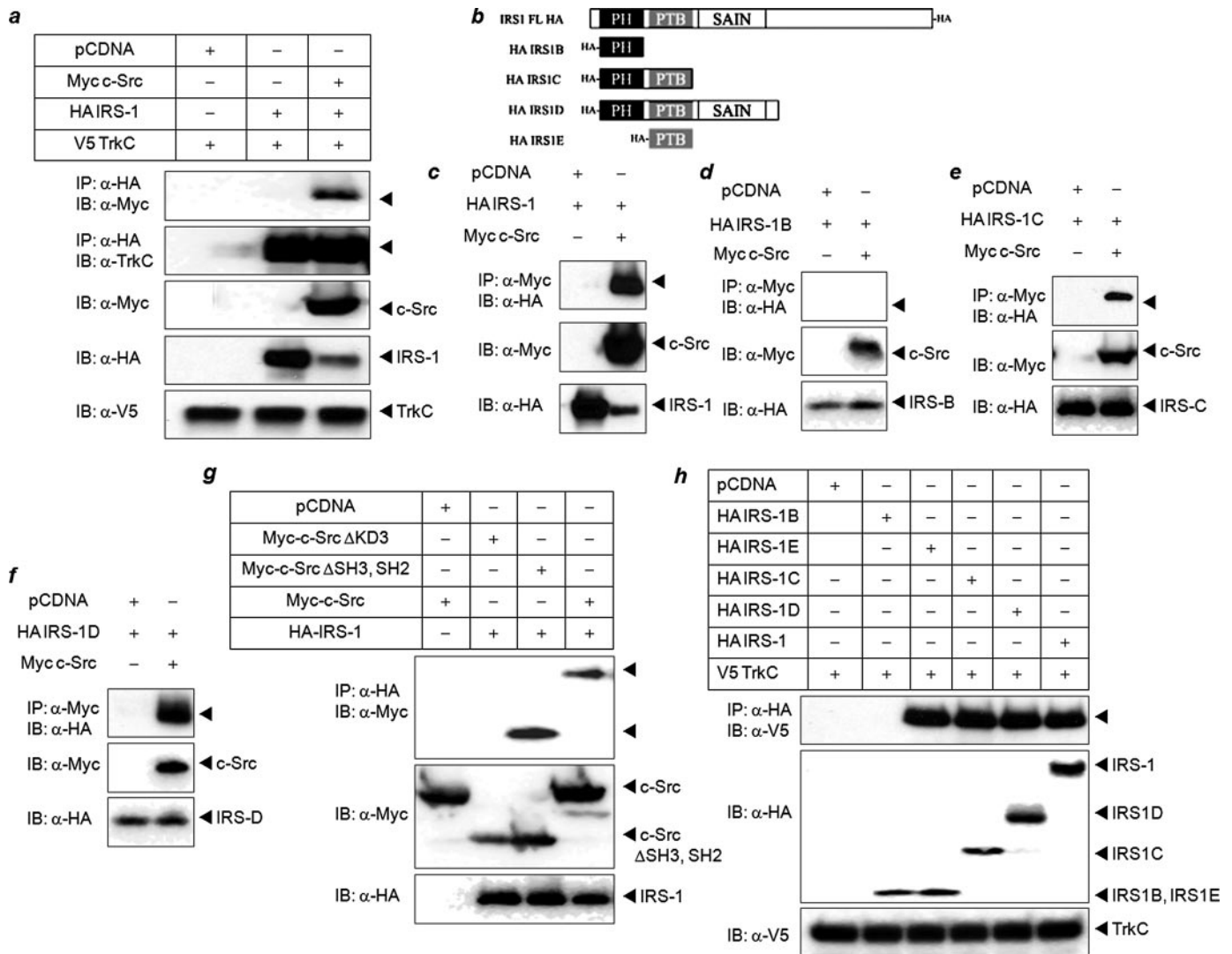
FIGURE 4. **TrkC interacts with c-Src and IRS-1.** *a*, V5-tagged TrkC was cotransfected into 293T cells with the HA-tagged IRS-1 and Myc-tagged c-Src constructs. Cell extracts were subjected to immunoprecipitation using anti-HA antibody and Gamma-bind beads, followed by immunoblotting with anti-V5 antibody. The expression of c-Src, IRS-1, or EN was monitored as indicated. *b*, schematic representation of full-length and truncated IRS-1 proteins is shown. *c–f*, PTB domain of IRS-1 is important for association with c-Src and TrkC. Myc-tagged c-Src was cotransfected into 293T cells with HA-tagged IRS-1. *g*, identification of c-Src domain interacting with IRS-1. *h*, IRS-1-truncated constructs and V5-tagged TrkC were cotransfected into 293T cells. Cell extracts were subjected to immunoprecipitation using anti-HA antibody, followed by immunoblotting with anti-V5 antibody.

not have a negative-regulatory tyrosine in the C-terminal region, still interacted with TrkC. While the deletion of amino acid residues 361–535 (ΔKD-1 and ΔKD-2) also interacted with TrkC, the deletion of amino acid residues 275–360 abrogated the TrkC interaction (Fig. 3*c*). These results clearly indicate that the region including amino acids 275–360 of c-Src, which includes the ATP binding domain, is required for TrkC interaction.

The Fyn tyrosine kinase, a member of the c-Src kinase family, is known to associate with IRS-1 *in vitro* and *in vivo* (53). Because other members of the c-Src kinase family have similar SH2 domains, these kinases may also associate with IRS-1 and contribute to the overall response in various cellular backgrounds. Because IRS-1 functions as the adaptor protein linking ETV6-NTRK3 (Tel-TrkC) to downstream signaling pathways (54), we examined whether the interaction between TrkC and c-Src is mediated through IRS-1. 293T cells were transfected with Myc-tagged c-Src and HA-tagged IRS-1 with or without

V5-tagged TrkC. IRS-1 interacts with either TrkC or c-Src (Fig. 4*a*). Three different domains in IRS-1 have been identified as potentially contributing to insulin-like growth factor I receptor (IGFIR) and insulin receptor binding: the pleckstrin homology (PH) domain, the phosphotyrosine binding (PTB) domain, and the SHC and IRS-1 NPXY binding (SAIN) domain (55). PH domains bind phospholipids, thereby mediating the interaction of signaling proteins such as IRS-1 with the plasma membrane (56). PTB domains in adaptor proteins bind to phosphorylated tyrosines within NP*X*Y motifs in interacting proteins such as cell surface receptors (57), thus promoting receptor-adaptor interactions. The SAIN domain of IRS-1 remains poorly characterized but has been postulated to contain other potential protein-protein interaction motifs (55, 58) (Fig. 4*b*). To determine the region of IRS-1 responsible for binding to c-Src, lysates were immunoprecipitated with Myc antibodies followed by immunoblotting with HA antibodies. Myc-tagged c-Src was able to interact with the IRS-1 construct containing the PTB
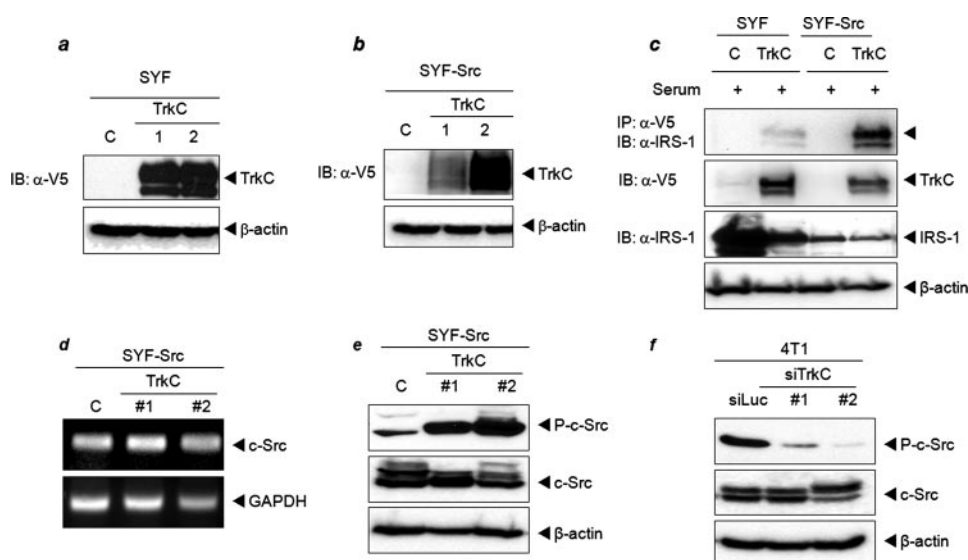
FIGURE 5. **c-Src expression in c-Src-deficient MEF cells enhances the TrkC-IRS-1 complex formation.** *a* and *b*, expression of TrkC protein was examined by immunoblotting in SYF and SYF-Src cells expressing EN. *c*, cell extracts were subjected to immunoprecipitation using anti-V5 antibody and Gamma-bind beads, followed by immunoblotting with anti-IRS-1 antibody. The expression of V5-TrkC or IRS-1 was monitored as indicated. *d*, expression of *c-src* mRNA in control and TrkC expressing SYF-Src cells was examined by RT-PCR. The endogenous *GAPDH* mRNA level was measured as an internal control. *e*, TrkC increased phosphorylation of c-Src *in vivo* in control and TrkC expressing SYF-Src cells. Cell lysates were probed for c-Src and phosphorylated c-Src as well as β-actin as a loading control. *f*, silencing of TrkC decreased c-Src phosphorylation. Phosphorylated c-Src and total c-Src proteins were examined by immunoblotting in 4T1 cells stably expressing control-siRNA (siLuc) and siTrkC-siRNA.

domain (HA-IRS-1C, HA-IRS-1D, and HA-IRS-1) (Fig. 4, *c–f*). Only the fragment consisting of the IRS-1 PH domain alone (IRS-1B) was incapable of associating with Myc-tagged c-Src, indicating that the PTB domain of IRS-1 is required for its interaction with c-Src. Also, to determine the region of c-Src responsible for binding to IRS-1, lysates were immunoprecipitated with HA antibodies followed by immunoblotting with Myc antibody. HA-tagged IRS-1 was able to interact with Myc-tagged c-Src constructs containing the C-terminal region (254–535) (ΔSH3,SH2) (Fig. 4*g*). We also attempted to determine the region of IRS-1 responsible for binding to TrkC. Lysates were immunoprecipitated with HA antibodies followed by immunoblotting with V5- antibodies. V5-tagged TrkC was able to interact with all IRS-1 constructs containing the PTB domain (HA-IRS-1C, HA-IRS-1D, and HA-IRS-1) (Fig. 4*h*), indicating that the PTB domain of IRS-1 is also required for its interaction with TrkC.

c-Src Expression in c-Src-deficient MEF Cells Enhances the TrkC-IRS-1 Complex Formation—To test whether TrkC binding to IRS-1 requires c-Src, we used SYF cells that were derived from Src, Yes, and Fyn triple knock-out mouse embryos (59). Because Src, Yes, and Fyn are three ubiquitously expressed members of the Src family tyrosine kinases, no Src family tyrosine kinase activity was detected in these SYF cells. We infected SYF and SYF-c-Src cells with the lentiviral vector transducing *TrkC* gene. As shown in Fig. 5, *a* and *b*, expression of TrkC protein in these cells was verified by immunoblotting using the anti-V5 antibody. We next determined whether TrkC-IRS-1 complex formation is increased in the presence of c-Src. IRS-1 and TrkC complexes were detected in c-Src-deficient SYF cells. However, the level of IRS-1 immunoprecipitated with TrkC

was markedly enhanced in SYF cells expressing c-Src compared with that in SYF cells (Fig. 5*c*), even though level of endogenous IRS-1 is significantly lower in SYF-c-Src cells compared with SYF cells. These results suggest that c-Src mediates the TrkC-IRS-1 interaction. To see whether IRS-1 is also required for transforming activity of 4T1 cells, the RNA interference approach was employed to knockdown endogenous IRS-1 in 4T1 cells. We constructed lentiviral vectors expressing two different IRS-1 siRNAs (1 and 2). To establish cell lines expressing these IRS-1 siRNA genes, two cycles of infection and selection were performed. Silencing of IRS-1 in 4T1 cells was confirmed by RT-PCR. Expression of IRS-1 siRNAs (1 and 2) resulted in over 80–90% decreases in the expression of endogenous IRS-1 (Supplemental Fig. S2*a*). The luciferase RNAi nucleotide (siLuc) was used as a control. Expression of IRS-1 siRNAs slightly decreased formation of microscopic or macroscopic colonies in soft agar when scored after 20 days, suggesting that IRS-1 may contribute to the transforming activity in 4T1 cells (Supplemental Fig. S2*b*).

To investigate whether TrkC modulated the c-Src expression, we examined the expression of c-Src in SYF-SRc cells expressing TrkC and 4T1 cells expressing siTrkC by RT-PCR. c-Src transcript was not affected by the expression of TrkC in SYF-Src cells, but c-Src protein expression was slightly decreased compared with the control cells (Fig. 5, *d* and *e*). However, c-Src phosphorylation levels were markedly increased in SYF-Src stably expressing TrkC cells, suggesting that TrkC activates c-Src in these cells (Fig. 5*e*). Consistent with this finding, we found that c-silencing of TrkC in 4T1 cells markedly decreased c-Src phosphorylation levels (Fig. 5*f*).

Effects of c-Src-targeted siRNA on Transformation of 4T1 Cells—We have recently demonstrated that inhibition of TrkC expression markedly suppresses the colony formation of 4T1 cells.[3] To see whether inhibition of c-Src is also required for transforming activity of 4T1 cells, the RNA interference approach was employed to knockdown endogenous c-Src in 4T1 cells. We constructed lentiviral vectors expressing two different c-Src siRNAs (1 and 2). To establish cell lines expressing these c-Src siRNA genes, two cycles of infection and selection were performed. The c-Src gene silencing activity of c-Src RNAi in 4T1 cells was confirmed by immunoblotting with c-Src antibodies. Expression of c-Src siRNAs (1 and 2) resulted in over 80% decreases in the expression of endogenous c-Src (Fig. 6*a*). The luciferase RNAi nucleotide (Si-Luc) was used as a control. In the previous study, we have shown that TrkC is responsible for the elevated activa-
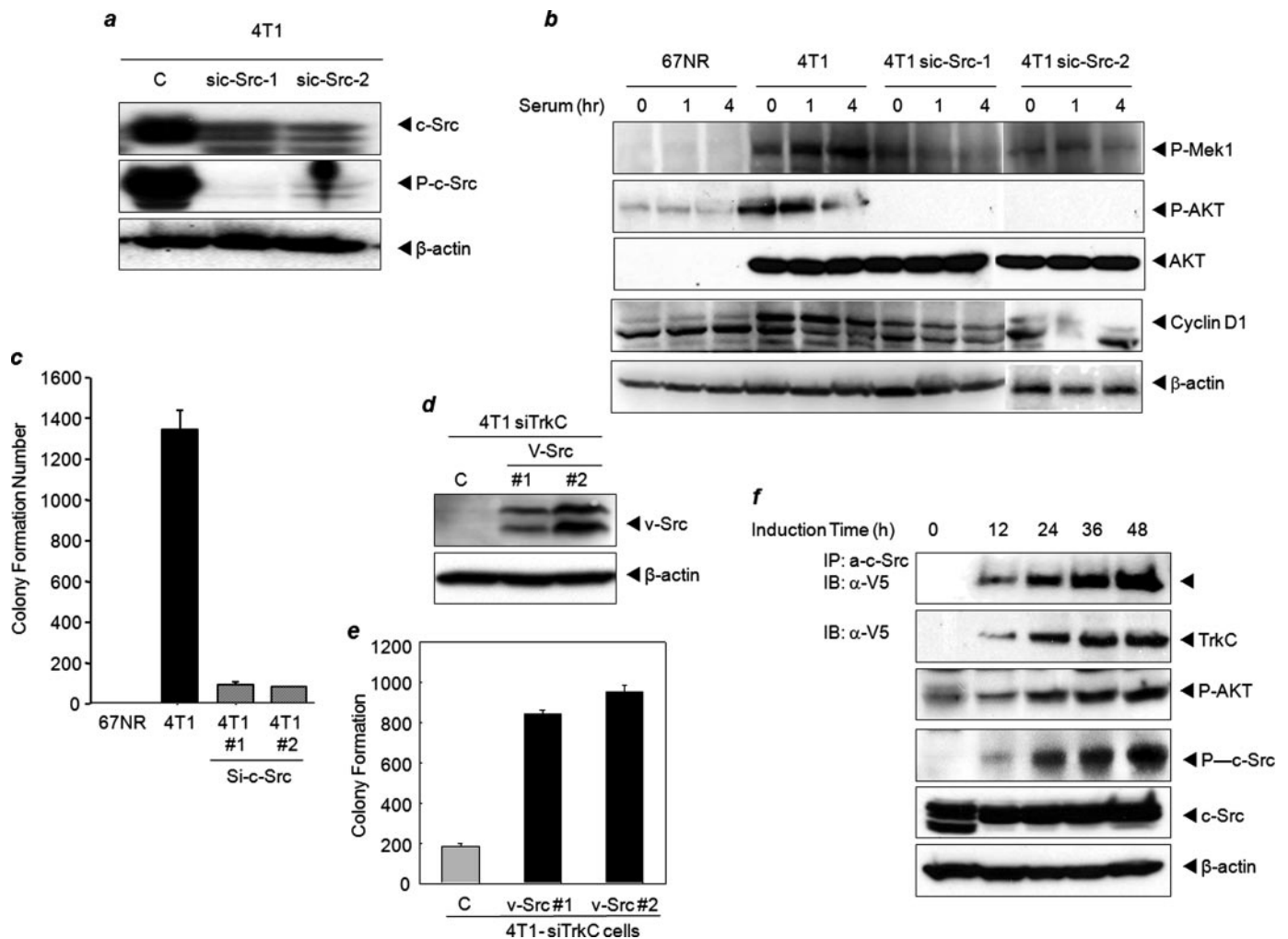
FIGURE 6. **Suppression of c-Src expression by stable siRNA reduces the level of MEK phosphorylation, AKT phosphorylation, and cyclin D1 expression in 4T1 cells.** *a*, suppression of c-Src expression by stable siRNA. Expression of phosphorylated c-Src and c-Src protein was examined by immunoblotting in 4T1 cells stably expressing control-siRNA, c-Src-siRNA-1, and c-Src-siRNA-2. *b*, suppression of c-Src expression by stable siRNA decreases levels of phosphorylated AKT, phosphorylated MEK, and cyclin D1 in 4T1 cells. c-Src-siRNA-expressing 4T1 or control 67NR cells were serum-starved overnight in 0.5% serum and then stimulated with and without 9% CS/DMEM for 1 or 6 h. Whole cell lysates were prepared for Western blotting and probed with antibodies against the phosphorylated forms of Mek1/2 (Ser$^{217/221}$), phosphorylated Akt (Ser^{473}), and cyclin D1. *c*, soft agar colony-forming assay. Soft agar colony-forming assay of 4T1 control-siRNA, 4T1 c-Src-siRNA-1, and EN c-Src-siRNA-2. Monolayer cells were trypsinized, washed, and plated in medium containing 0.2% agar to assess anchorage-independent growth. Results are presented as the number of macroscopic colonies formed at 3 weeks after plating. *d*, expression of v-Src was examined by immunoblotting in 4T1-siTrkC cells stably expressing control and v-Src. *e*, v-Src reverses the effect of TrkC knockdown on colony forming ability of 4T1 cells. Soft agar colony-forming assay of 4T1-siTrkC cells expressing either control or v-Src. Results are presented as the number of macroscopic colonies formed at 3 weeks after plating. *f*, induction of TrkC expression by doxycycline increased the level of c-Src bound to TrkC in T-47D tet on cells. Cells were cultured for 0–48 h in the presence (2 μg/ml) and absence of doxycycline. Cell extracts were subjected to immunoprecipitation using anti-c-Src antibody or anti-mouse IgG, followed by immunoblotting with anti-V5 antibody. Expression of V5-TrkC, c-Src, phosphorylated c-Src, and phosphorylated AKT was monitored as indicated.

tion of the Mek1 and Akt in 4T1 cells. Therefore, we examined whether c-Src activation is involved in the TrkC-induced Mek1 and Akt activation as well as cyclin D1 expression. As shown in Fig. 6*b*, TrkC-induced Mek1 and Akt activation as well as cyclin D1 expression were significantly reduced in c-Src knockdown cells. Expression of c-Src RNAi nucleotides (Si-c-Src) failed to form microscopic or macroscopic colonies in soft agar when scored after 16 days (Fig. 6*c*). We then examined whether v-Src reverses the effect of TrkC knockdown on colony forming ability of 4T1 cells. To do so, we infected 4T1-siTrkC cells with the recombinant lentiviral vector carrying a *v-Src* cDNA or empty vector as a control. Expression of the predicted v-Src protein in transformed 4T1-siTrkC cells was verified by immunoblot-

ting using the anti-V5 antibody (Fig. 6*d*). Expression of v-Src markedly increased colony formation in 4T1-siTrkC cells (Fig. 6*e*), suggesting that c-Src plays an important role in TrkC-induced colony formation.

To see the whether increasing levels of TrkC further activates Akt, we transfected TrkC in T-47D tet-on cell lines and examined the Akt activation. We found that levels of activated Akt correlated with level of TrkC expression. We also demonstrated that levels of c-Src associated with TrkC and levels of phosphorylated c-Src were also increased when TrkC expression was induced (Fig. 6*f*). Taken together, our data suggest that c-Src plays a role in the TrkC-induced MEK1/2 and Akt activation and cyclin D1 expression and mediates the TrkC transformation activity.
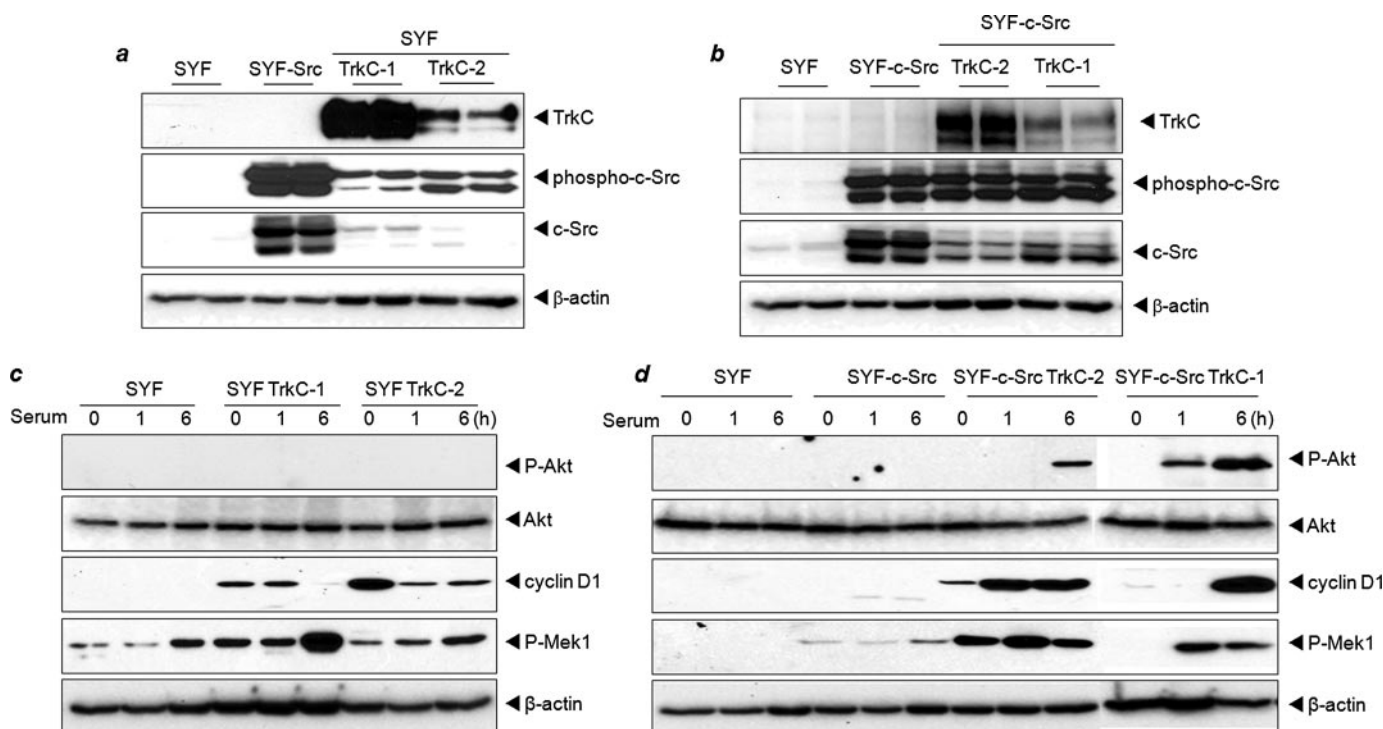
FIGURE 7. **c-Src is required for TrkC-induced Akt activation in Src-deficient MEF cells.** *a* and *b*, to generate SYF or SYF-c-Src cell lines stably expressing TrkC were infected with lentiviruses containing control vector alone (pCAG) or TrkC. Successful expression of TrkC was determined using anti-TrkC antibody. TrkC-expressing SYF and SYF-c-Src cells exhibit elevated levels of phosphorylated Src. *c*, TrkC-expressing SYF cells exhibit elevated levels of phosphorylated Mek1 and cyclin D1, but not phosphorylated Akt. *d*, TrkC-expressing SYF-c-Src cells exhibit elevated levels of phosphorylated Mek1, phosphorylated Akt, and cyclin D1. TrkC-expressing or control SYF cells and TrkC-expressing or control SYF-c-Src cells were serum-starved overnight in 0.5% serum and then stimulated with (+) and without (−) 9% calf serum/DMEM for 1 or 6 h. Whole cell lysates were prepared for Western blotting and probed with antibodies against the phosphorylated forms of Mek1/2 (Ser$^{217/221}$), phosphorylated Akt (Ser^{473}), cyclin D1, and β-actin (as a loading control). Data are representative of three experiments.

c-Src Is Required for TrkC-induced Mek1 and PI3K/Akt-1 Activation and Cyclin D1 Expression in MEF Cells—To test the role of c-Src on the Mek1 and PI3K-Akt pathways in TrkC signaling, we investigated the activation of Mek1 and PI3K-Akt by TrkC in SYF and SYF-c-Src cells. To do so, we constructed a lentiviral vector expressing the TrkC. To establish cell lines expressing this TrkC gene, two cycles of infection and selection were performed. TrkC expression in SYF and SYF-c-Src cells was confirmed by immunoblotting with TrkC antibody. SYF cells and SYF-c-Src cells with or without TrkC were serum-starved overnight and then incubated with serum. The phosphorylated c-Src was only detected in SYF-c-Src cells (Fig. 7, *a* and *b*). Interestingly, in SYF cells expressing TrkC, phosphorylated proteins recognized by anti-phospho-c-Src antibody were detected. This suggests that other c-Src family members, which are also recognized by anti phospho-c-Src antibody, may be activated by TrkC.

TrkC signaling is known to induce phosphorylation of the Ras/MAPK and PI3K/Akt (60). To elucidate which intracellular pathways could be involved in c-Src-mediated TrkC signaling, we assessed the degree of activation of the Ras/MAPK and PI3K/Akt pathways in SYF and SYF-c-Src cells expressing TrkC. Cells were assayed before and after serum stimulation for Mek1 and Akt activation by Western blot analysis of whole cell lysates using antibodies directed against the phosphorylated (activated) forms of these antibodies. As shown in Fig. 7*c*, Mek1 and PI3K/Akt activation as well as cyclin D1 expression were

not observed in SYF cells. TrkC expression did not activate Akt in SYF cells. However, stable expression of TrkC in SYF-c-Src cells led to Akt phosphorylation in response to serum stimulation, suggesting that TrkC-induced PI3K-Akt activation requires c-Src (Fig. 7*d*). In both SYF and SFY-c-Src cells, expression of TrkC increased Mek1 phosphorylation even in the absence of serum (Fig. 7, *c* and *d*). SYF cells expressing TrkC exhibited constitutively high levels of cyclin D1 even after overnight serum starvation (Fig. 7*c*). SYF-c-Src cells responded to TrkC, showing cyclin D1 elevation in the presence of serum (Fig. 7*d*). These genetic data demonstrated that c-Src is required for TrkC-induced PI3K/Akt activation.

DISCUSSION

The results of this study demonstrate that c-Src mediates TrkC-induced activation of the PI3K/Akt pathway. TrkC directly interacts with c-Src. Knockdown of c-Src suppressed activation of the PI3K/Akt pathway and markedly decreased the transforming activity of 4T1 cells which express endogenous TrkC. Finally, we also demonstrated that TrkC failed to activate the PI3K/Akt pathway in c-Src-deficient SYF cells.

The Trk family of neurotrophin receptors is emerging as an important player in carcinogenic progression. Trks appear to have a high capacity for ligand-independent activation, presumably via spontaneous interactions. Recent studies have identified correlations between biological responses and the activation of selective intracellular signaling pathways medi-

ated by TrkC (60). TrkC is known to activate intracellular signaling cascades, including the Ras/Erk pathway and the PI3K/Akt pathway (60–62). The Ras/Erk pathway plays an essential role in neuronal differentiation. In contrast, the PI3K/Akt pathway is essential for progenitor survival. These findings suggest that both Ras/Erk pathway and the PI3K/Akt pathway play a significant role in TrkC-mediated oncogenesis. This hypothesis is also supported by a previous study of ETV6-NTRK3 (Tel-TrkC) (42). Both the Ras/Erk and PI3K/Akt pathways appear to be essential for transformation and cell cycle progression in ETV6-NTRK3-expressing cells. Aberrant activation of the Ras/Erk pathway is known to be associated with cellular transformation (63, 64). Alterations in the PI3K-AKT pathway have also been observed in humans tumors (65, 66). Although these and other studies illustrate the oncogenic potential of each individual pathway, it is becoming increasingly clear that a synergistic effect exists between the Ras-Erk1/2 and PI3K-Akt cascades in transformation. In the present investigation, we found that c-Src is required for TrkC-induced activation of the PI3K/Akt pathway. These data place c-Src upstream of PI3K/Akt and provide a mechanism of TrkC-induced cellular transformation.

All Src family members are composed of several well-characterized protein domains. c-Src is a multidomain protein and consists of an N-terminal membrane targeting signal, a SH3 domain, a SH2 domain linked to the kinase domain, and a C-terminal tail (67). The catalytic domain is well conserved in all members of the c-Src family. The catalytic domain, which is responsible for the catalytic activity of the molecule, contains many subdomains that are closely related to the serine-threonine kinases, including the ATP-binding pocket. Analysis of a series of c-Src deletion mutants for TrkC binding domain revealed that amino acids 274–360 of c-Src is responsible for TrkC binding. This association is presumed to initiate c-Src activation, by allowing the molecule to adopt the catalytically active conformation. However, this hypothesis requires further experiments for validation. Recent studies suggest that the activation of the Src family tyrosine kinases by receptor tyrosine kinases may be more complex than simple recruitment. For example, the activation of c-Src by epidermal growth factor receptor in transfected cells requires the small GTPases Ras and Ral (68). There is also a role for the tyrosine phosphatase Shp2 in promoting the activation of Src family tyrosine kinases in response to growth factors (69). A more recent report suggests that Shp2 may be involved in TrkC-induced c-Src activation (70). The fibroblast growth factor (FGF) receptor substrate 3 (FRS3) binds all neurotrophin Trk receptor-tyrosine kinases and becomes tyrosine-phosphorylated in response to NGF, BDNF, and NT-3 stimulation. The signaling molecules Grb2 and Shp2 bind FRS3 and Shp2, in turn, becomes tyrosine-phosphorylated.

IRS-1 contains 21 putative tyrosine phosphorylation sites, several of which are located in amino acid sequence motifs that bind to SH-2 domain proteins, including the p85 regulatory subunit of PI 3-kinase, Grb-2, Nck, Crk, Fyn, Csk, phospholipase Cγ, and Shp2 (55–58). IRS-1 contains also >30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases such as casein kinase II, protein kinase C, PI3K/Akt, and MAP kinases. In this report, we have shown that the

C-terminal region of c-Src interacts with IRS-1. However, it remains to be clarified whether the TrkC binding domain in c-Src overlaps with the IRS-1 binding domain in c-Src. We also cannot exclude the possibility that c-Src mediates complex formation between IRS-1 and TrkC. This hypothesis is supported by the finding that the level of IRS-1 immunoprecipitated with TrkC was markedly enhanced in SYF cells expressing c-Src compared with that in c-Src-deficient SYF cells (Fig. 5c). Another possibility is that TrkC may directly, but weakly, interact with IRS-1. It is possible that an unidentified protein may also be involved in the TrkC-IRS-1 complex formation.

Alterations in the PI3K/AKT pathway have also been observed in humans tumors, including amplification of the AKT β isoform in breast and ovarian carcinomas (71) and amplification of the PI3K regulatory subunit in ovarian carcinoma (66) Although these and other studies (reviewed in Ref. 20) illustrate the oncogenic potential of each individual pathway, it is becoming increasingly clear that a synergistic effect exists between the Ras/MAPK and PI3K/Akt cascades in transformation. Recent studies suggest that depletion of endogenous c-Src levels by means of siRNA or expression of dominant-negative c-Src significantly reduced cell proliferation *in vitro*, and led to a clear decrease in Akt phosphorylation, placing c-Src upstream of the PI3 kinase/Akt signals. Taken together, our observations establish an essential role for c-Src kinase in activation of the PI3K/Akt cascade in TrkC-expressing cells.

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